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NOTES

Infectious Particles Derived from Semliki Forest Virus Vectors Encoding Murine Leukemia Virus Envelopes

IRINA LEBEDEVA,† KAZUNOBU FUJITA,‡ ABDALLAH NIHRANE,§ AND JONATHAN SILVER*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

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Semliki Forest virus vectors encoding murine leukemia virus (MLV) envelope protein with a truncated cytoplasmic tail generate submicrometer, cell-associated, membranous particles that transmit replication-competent vector RNA specifically to cells bearing the MLV receptor. Such "minimal" viruses could have applications as retroviral vaccines or in the study of virus evolution.

Semliki Forest virus (SFV) contains a single-stranded, ~11-kb positive-sense RNA, the first two-thirds of which encodes nonstructural proteins involved in viral replication, while the last third encodes the capsid and envelope (for a review, see reference 20). Vectors have been made from SFV (and the closely related Sindbis virus) by deleting capsid and *env* sequences (2, 4, 10, 18, 23). RNA transcribed in vitro from a plasmid containing vector sequence downstream of a bacterial RNA promoter can be introduced into mammalian cells by electroporation or lipofection. The vector RNA is translated to produce enzymes which replicate vector RNA to high levels, usually shutting off host cell synthesis and causing cell death in 1 to 3 days. Passenger genes, inserted into these vectors downstream of an internal promoter that normally drives production of capsid and envelope, can be expressed at high levels (10, 18).

Replicase-negative vectors can also be made that lack the replicase genes but retain *cis*-active sequences at the 5′ and 3′ termini required for replication (10). Such vectors do not replicate by themselves but will replicate in cells that express the viral replicase proteins. One such vector encoding SFV capsid-Env has been used as a packaging system (9a). SFV capsid-envelope is synthesized as a polyprotein, the capsid portion of which has an endoprotease activity that cleaves itself off of the precursor (20). Foreign genes can be inserted in frame just downstream of the capsid in such a way that the capsid cleaves itself off of the fusion protein (18). The normal SFV capsid-envelope complex forms particles that selectively package SFV RNAs containing a packaging sequence thought to overlap replicase genes between bases 2737 and 2993 (20).

Rolls et al. (15) reported that cells containing an SFV vector encoding the vesicular stomatitis virus (VSV) envelope glycoprotein (G protein) produced submicrometer particles that could transfer vector RNA to new cells via the fusogenic activity of VSV G protein. The vector RNA replicated in newly infected cells and spread to a limited extent in tissue culture. Similar particles were formed when rabies virus envelope was substituted for VSV G protein, although the yield of infectious particles was lower (15).

We were interested to see if a similar phenomenon could be established by using a retroviral envelope. While studies performed several years ago with avian retroviruses (ALV) indicated that ALV envelopes did not pseudotype Sindbis virus particles (24), the results of Rolls et al. (15) raised the possibility that retroviral envelopes might replace the SFV envelope in a more primitive virus vector system.

For safety reasons, we began by using a double-vector SFV system in which replicase genes and capsid-envelope genes are encoded on different RNAs. Starting with a replicase-negative vector encoding SFV capsid-Env, we replaced SFV *env* sequences with *env* sequences from Moloney murine leukemia virus (MLV) to make the vector S*cap*M*env* (Fig. 1, upper panel, construct 1). The MLV Env is synthesized as a gp85 precursor that is normally cleaved into a 70-kDa surface (SU) glycoprotein (gp70) and a 15-kDa transmembrane (TM) moiety (p15e). In a late maturation step mediated by retroviral protease, the cytoplasmic tail of MLV p15e is cleaved to make a p12e TM species (17). This cleavage increases the fusogenicity of MLV Env (14). To mimic this activating cleavage of p15e, we made a version of the S*cap*/M*env* vector in which the TM was truncated by placing a stop codon at the p12e cleavage site (S*cap*M*env*-tr; Fig. 1, upper panel, construct 2).

Since the above vectors encode SFV capsid and SFV capsid interacts with the ~30-amino-acid cytoplasmic tail of the SFV E2 envelope protein in forming infectious particles (11, 19, 22), we also made vectors encoding chimeric envelopes in which the cytoplasmic tail of SFV E2 (amino acids 3197R to 3227A) was appended to the carboxy terminus of the MLV TM (S*cap*M*env* SE2 and S*cap*M*env*-trSE2; Fig. 1, upper panel, constructs 3 and 4). Exact sequence joints were made by PCR overlap extension (7).

Replicase-positive SFV vectors encoding Moloney MLV Env with the full-length or truncated cytoplasmic tail were constructed by inserting these sequences into pSFV1 (Fig. 1, lower panel, second and third constructs). To indicate that these vectors contain the SFV replication functions, we refer to them as S*rep*M*env* and S*rep*M*env*-tr, respectively. In some experiments we used an SFV vector encoding β-galactosidase

* Corresponding author. Phone: (301) 496-3653. Fax: (301) 402-0226. E-mail: Jsilver@nih.gov.

† Present address: Division of Oncology, Department of Medicine, Columbia University, New York, N.Y.

‡ Present address: Department of Pediatrics, Showa Medical School, Tokyo, Japan.

§ Present address: Hôpital St. Vincent de Paul, INSERM, Paris, France.

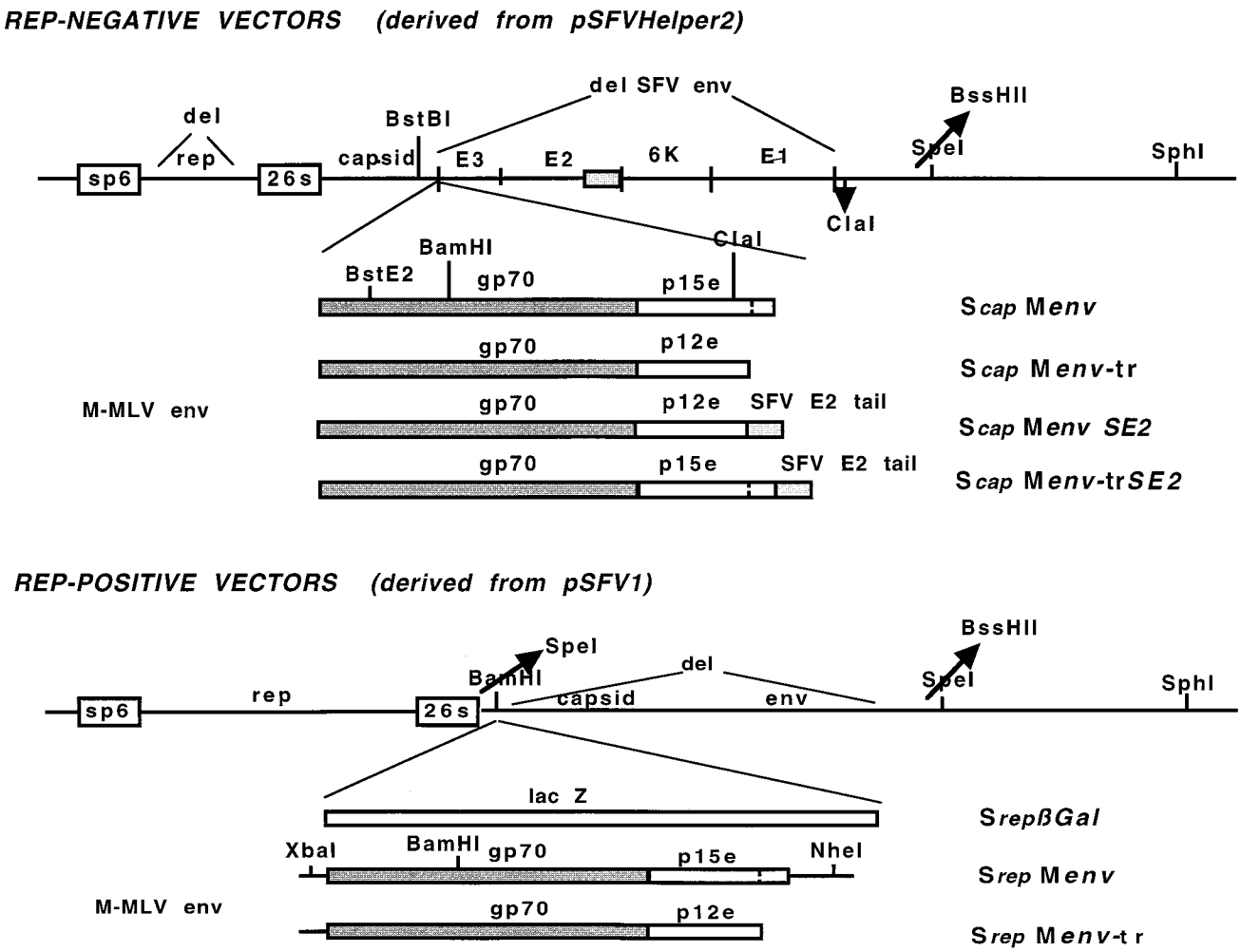

FIG. 1. Diagram of SFV vectors used in this study. The parent vectors pSFV1, pSFVhelper2, and pSFVLacZ were obtained from Life Technologies (9a), and MLV sequences were derived from the Moloney MLV clone p63-2 (1), a gift from Hung Fan (University of California at Irvine). sp6, RNA polymerase promoter used for in vitro transcription of vector RNA; 26s, internal SFV promoter for subgenomic RNA species; rep, SFV replicase (nonstructural) genes; cap, SFV capsid; env, SFV or MLV envelope; E3, E2, 6K, and E1 are portions of the SFV envelope; gp70 and p15e/p12e are portions of MLV envelope; del, deletion; S, SFV; M, Moloney MLV; tr, truncated; lacZ, βgal. Details of construction are available on request.

(βgal) (SFV3LacZ) (9a), which for consistency we refer to as S*rep*β*gal*.

Expression of vector RNA. When BHK cells were electroporated with replicase-positive SFV vectors, vector RNAs replicated to high levels and vector RNA species were visible on ethidium-stained gels. Northern blot hybridization with an SFV probe confirmed that the new RNA species contained vector sequences (not shown). When replicase-negative SFV vectors encoding SFV capsid were coelectroporated with replicase-positive pSFV1, RNA derived from the replicase-negative vectors was detectable by using an SFV capsid probe (not shown).

Expression of vector-encoded Env proteins. Polyclonal anti-MLV Env SU antiserum immunoprecipitated an ~85-kDa protein from pulse-labeled BHK cells electroporated with vectors encoding (i) full-length MLV Env (Fig. 2, S*rep*M*env* and S*cap*M*env* lanes), (ii) MLV Env with a truncated cytoplasmic tail (S*cap*M*env*-tr lane), or (iii) MLV Env with a cytoplasmic tail derived from the SFV Env E2 protein (S*cap*M*env*SE2 lane). Replicase-negative vectors in this experiment were coelectroporated with S*rep*β*gal* to provide replicase functions. The ~85-kDa protein was absent in negative control BHK cells electroporated with S*rep*β*gal* alone (lane None) or S*rep*β*gal* plus a vector encoding SFV Env (S*cap*S*env* lane). The size of the "gp85" was slightly smaller in the case of the 16-amino-acid cytoplasmic tail truncation (S*cap*M*env*-tr lane) and slightly larger in the case of the appended 31-amino-acid cytoplasmic tail from SFV E2 (S*cap*M*env*SE2 lane). The vector encoding the truncated MLV Env with the cytoplasmic tail of SFV Env E2 appended to its carboxy terminus produced little or no Env protein (S*cap*M*env*-trSE2 lane). The fact that the MLV Env species had a molecular size of ~85 kDa in the case of the vectors that encoded MLV Env as a fusion protein with SFV capsid suggests that the capsid had cleaved itself off the fusion protein; otherwise the 238 amino acids of SFV capsid should have made the fusion protein more than 100 kDa. In other immunoprecipitations, we detected an ~30-kDa species (presumably SFV capsid) specifically in cells electroporated with S*cap* vectors.

In MLV-infected cells, the gp85 Env precursor is cleaved into a gp70 SU and a p15E TM species. Pulse-chase experiments in BHK cells electroporated with S*cap*M*env* showed that

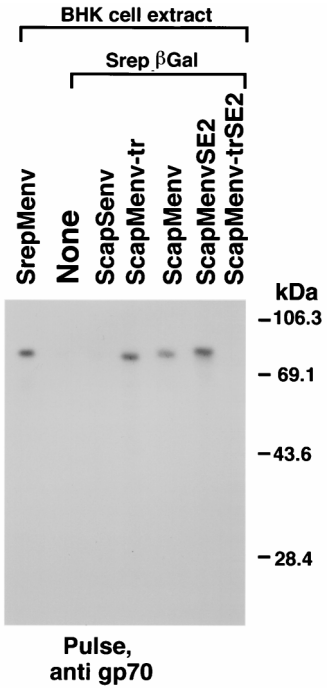

FIG. 2. Vector-encoded envelope proteins analyzed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. BHK cells were electroporated 20 h previously with the indicated vector RNAs, metabolically labeled for 30 min with [^{35}S]Met and [^{35}S]Cys, and immunoprecipitated with polyclonal goat anti-MLV SU (gp70) Env antiserum (Quality Biotech, Camden, N.J.).

this cleavage was less extensive and the cleavage product was larger, ~80 kDa (not shown). The ~80-kDa species could reflect an aberrantly glycosylated form of MLV Env, because aberrant mobility of MLV Env due to altered glycosylation has been reported in other cells (8). Reduced cleavage of MLV gp85 could be due to the fact that this cleavage is performed by a cellular protease and SFV vectors shut down host cell protein synthesis (10). Cleavage of the gp85 Env precursor was confirmed by detection of TM species using an anti-TM monoclonal antibody (not shown). Suomalainen and Garoff (21) also found that the cleaved SU moiety was difficult to detect in BHK cells electroporated with an SFV vector expressing MLV Env, which they postulated was due to rapid degradation of SU in their system.

Fusogenic activity of vector-encoded MLV Env. When BHK cells were electroporated with vectors encoding full-length or truncated MLV Env and then cocultivated with XC cells, numerous syncytia were detected (Fig. 3A). When electroporated BHK cells were cocultivated with NIH 3T3 cells instead of XC cells, the vector encoding Env with the truncated cytoplasmic tail led to syncytium formation, whereas the vector expressing the full-length MLV Env did not (not shown). This result is not surprising since murine retroviruses encoding MLV Env with the truncated cytoplasmic tail form syncytia in NIH 3T3 cells as well as XC cells, whereas wild-type MLV Env forms syncytia only in XC cells (14).

Infectious particle formation. To assay for infectious particles, we coelectroporated BHK cells with the SFV vector encoding βgal as an indicator gene (S*rep*β*gal*) plus a replicase-negative SFV vector encoding MLV Env or SFV Env. Supernatants or dounce homogenates of these cells were passed through 0.45-μm filters and then used to infect a variety of target cells. One day later the target cells were stained for βgal activity.

Filtered supernatants from BHK cells coelectroporated with S*rep*β*gal* plus S*cap*S*env* (positive control) transmitted βgal to all cell types, as expected (Fig. 3C and Table 1, first row of each experiment). The SFV *env* in this vector carries several point mutations introduced for safety reasons that block processing of SFV Env by a cellular protease (9a). Treatment of these particles with chymotrypsin, which cleaves the SFV Env, increased titers ~10^3-fold (Table 1, experiment II), as expected (9a).

Filtered supernatants from BHK cells coelectroporated with SFV βgal plus S*cap*M*env*-tr transmitted βgal to NIH 3T3 cells (Fig. 3D and E), with titers of ~10^3 infectious units/ml. The βgal titer increased 10- to 100-fold when the BHK cells were mixed with their supernatants and dounce homogenized before filtration, indicating that most infectious particles were cell-associated or created during the process of homogenization. In contrast, dounce homogenization had no effect on the titer of particles derived from the S*cap*S*env* vector, indicating that these particles were efficiently released from cells.

Infectious particles infect via interaction with the MLV ecotropic receptor. While infectious particles derived from the S*cap*S*env* vector had a wide host range, the host range of S*cap*M*env*-tr particles paralleled that of MLV. Thus, they transmitted βgal to NIH 3T3 cells, which have a functional ecotropic MLV receptor, but not to NIH 3T3 cells chronically infected with Friend MLV (FV-NIH3T3) in which the receptor is blocked by interfering Friend MLV Env or to *Mus dunni* cells which bear a variant ecotropic receptor that is not susceptible to Moloney MLV (5) (Table 1, experiment I, row 2, and experiment II, row 3). The titer of these particles was also low on BHK cells, which do not have the ecotropic receptor, and was about 10^4 times lower in CHO cells than in CHO cells transduced with the ecotropic receptor (a gift from Carolyn Wilson, Center for Biologics Research, FDA, Bethesda, Md.) (Table 1, compare columns 3 and 2).

βgal was not transmitted by homogenates of BHK cells electroporated with S*rep*β*gal* alone (Fig. 3B and Table 1, experiment I, row 4) or S*rep*β*gal* plus the vector encoding full-length MLV Env, S*cap*M*env* (Table 1, experiment I, row 3). These results are consistent with infection being mediated by the ecotropic receptor and indicate that MLV Env with the full-length cytoplasmic tail does not interact with the receptor in a way that introduces vector RNA into cells.

Physical properties of infectious particles. The infectious, βgal-transducing activity was resistant to treatment with RNase (Table 2, experiment I, compare rows B and A), pelletable (experiment I, rows C and D, and experiment II, row B), and inactivated by 0.1% Triton (experiment II, rows C and D). These results are consistent with βgal vector RNA being packaged inside membrane-bound vesicles. Sucrose gradient analysis showed that the infectious particles had a density of 1.09 to 1.13 g/ml, similar to the particles reported by Rolls et al. (15). βgal infectious particles made with SFV capsid and SFV Env also had a density of 1.09 to 1.13 g/ml, slightly less than the density of MLV retroviruses (~1.15 g/ml).

Syncytium formation. Infection of NIH 3T3 cells with filtered homogenates from cells electroporated with S*cap*M*env*-tr plus S*rep*β*gal* led to syncytium formation in approximately 1 in 50 βgal-positive cells (Fig. 3D and E). Syncytia were not seen when the inoculum was derived from the S*cap*S*env* vector (Fig. 3C), consistent with syncytia resulting from expression of the fusogenic MLV Env. Syncytia in these experiments were only seen in cells positive for βgal, which is expected since the fusogenic MLV Env was encoded by a repli-

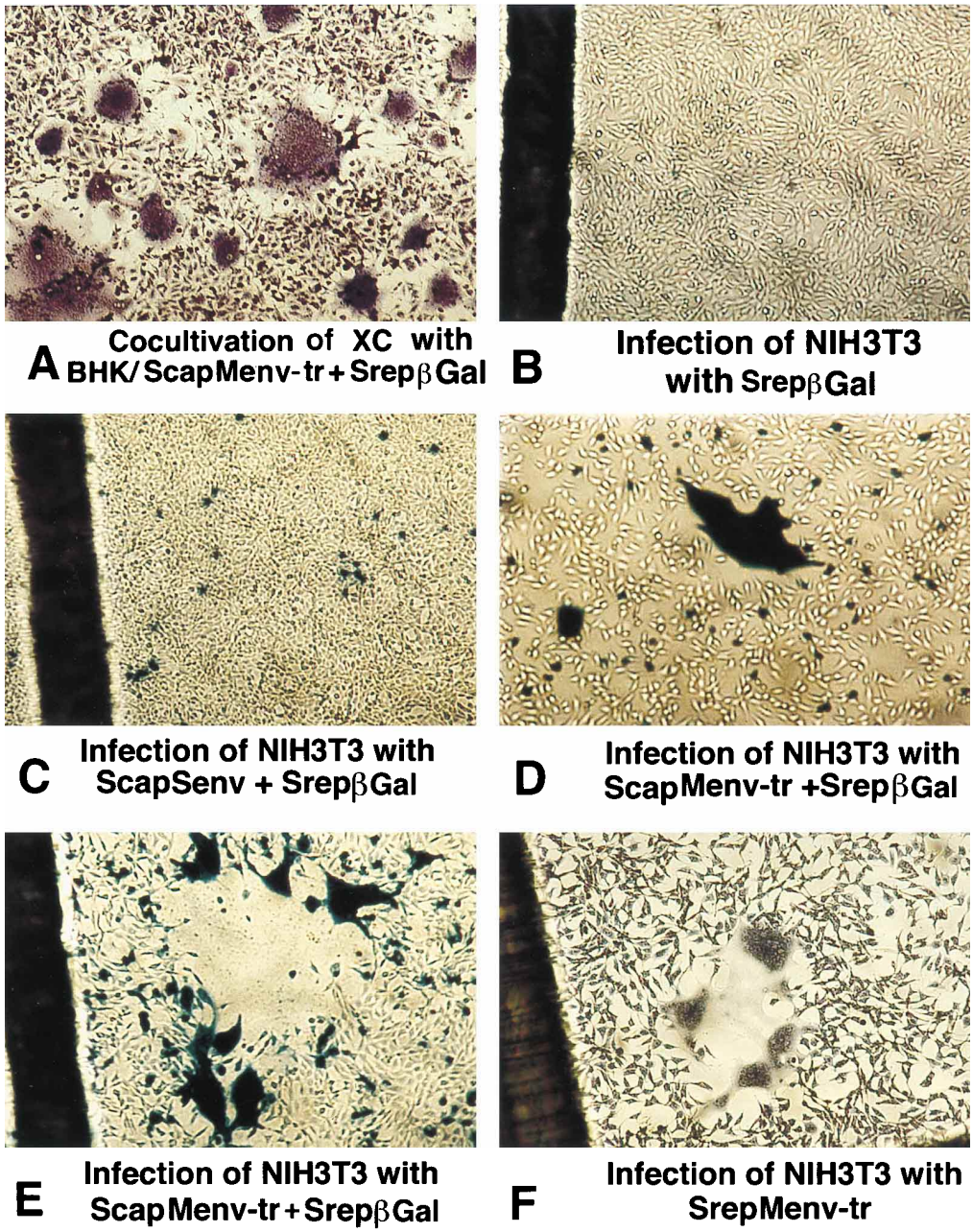

FIG. 3. Syncytium formation and βgal expression in cells electroporated or infected with SFV vectors. (A and F) Cells stained for syncytia with methylene blue-carbol fuchsin (0.85 g of carbol fuchsin and 2.5 g of methylene blue in 500 ml of methanol). (B through E) Cells stained for βgal, as described previously (9a). (A) XC cells cocultivated with BHK cells electroporated with S*cap*M*env*-tr. (B through F) NIH 3T3 cells infected with 0.45-μm-filtered homogenates of BHK cells electroporated with S*rep*β*gal* (B), S*rep*β*gal* plus S*capSenv* (C), S*rep*β*gal* plus S*cap*M*env*-tr (D and E), or S*rep*M*env*-tr (F).

case-negative vector and would only be expressed in cells that also contained SFV βgal RNA to supply the replicase genes.

When infected NIH 3T3 cells were monitored over time, small syncytia (the size of two or three cells) were detected 7 h after infection, becoming larger (about 5 to 10 cell diameters) after 24 h. Sometimes, plaques were seen containing rings of syncytia surrounding a central area devoid of cells (Fig. 3E and F), suggesting spread of vector with death of older infected cells. When NIH 3T3 cells infected with vectors encoding the truncated MLV Env were maintained in culture, they continued to produce syncytia for at least eight passages, but the number and size of the plaques did not increase, indicating some limitation to spread in culture.

Evidence for packaging of more than one vector RNA in single particles. There are several possible explanations for the fact that some infected NIH 3T3 cells produced both βgal and fusogenic MLV Env. One is that these cells were infected with recombinant vector RNAs encoding MLV Env plus βgal and SFV replicase. We feel this is unlikely because the ratio of syncytia to single βgal-expressing cells (about 1:50) is quite high for recombination, which has been estimated to occur at a frequency of 1 per 10^4 cells coelectroporated with SFV vec-

TABLE 1. Number of cells staining for βgal after infection of the indicated cell type with 1 ml of 0.45-μm-filtered homogenate from BHK cells coelectroporated with S*rep*β*gal* plus the indicated replicase-negative vector

Replicase-negative vector	Cell type (MLV receptor status)					
	BHK (−)	CHO (−)	ecoR-CHO (+)	NIH (+)	FV-NIH (−)	*M. dunni* (−)
Expt I						
S*cap*S*env* + CT[a]	2×10^4	8×10^3	5×10^3	5×10^3	10^4	ND[c]
S*cap*M*env*-tr	15	4	3×10^4	5×10^4	1	14[b]
S*cap*M*env*	8	6	30	3	1	ND
None	0	2	30	2	10	ND
Expt II						
S*cap*S*env* + CT[a]	2×10^6	6×10^3	1×10^4	7×10^3	3×10^4	2×10^3
S*cap*S*env* − CT	170	8	16	2	6	6
S*cap*M*env*-tr	46	0	8×10^3	4×10^4	8	0

[a] This stock was made from filtered supernatant (as opposed to homogenate) of electroporated BHK cells. CT, chymotrypsin.
[b] This datum point is from the results of a different experiment in which the S*cap*M*env*-tr homogenate had a titer of 4×10^4/ml on NIH 3T3 cells and 4/ml on FV-NIH 3T3 cells.
[c] ND, not done.

tors (9a). Also, recombination would be expected to lead to RNAs encoding SFV replicase plus MLV Env without βgal, yet no non-βgal-containing syncytia were observed.

The βgal-positive syncytia could also arise from infection of single NIH 3T3 cells with two particles, one containing SFV βgal RNA and the other containing S*cap*M*env*-tr RNA. In this case, the number of βgal-positive syncytia should decrease with the square of the dilution of the inoculum (two-hit kinetics). Alternatively, βgal-positive syncytia could arise from infection with single particles containing both RNAs, in which case the number of βgal-positive syncytia should decrease linearly with dilution (single-hit kinetics). The number of βgal-positive syncytia decreased linearly as the inoculum was diluted, strongly favoring the hypothesis that they resulted from infection with single particles containing two RNAs.

SFV capsid is not necessary for formation of infectious particles. The S*cap*M*env* vectors described above encode MLV Env as a fusion protein with SFV capsid. To see if capsid was necessary for infectious particle formation, we replaced the full-length MLV Env in S*rep*M*env* with the p12e stop codon-containing version. This vector, designated S*rep*M*env*-tr, is replicase positive and does not encode SFV capsid. Filtered homogenates of BHK cells electroporated with S*rep*M*env*-tr RNA induced syncytia in NIH 3T3 cells (Fig. 3F) but not in FV-NIH3T3 cells or in *M. dunni* cells, consistent with particles infecting via the ecotropic retroviral receptor. Thus, SFV capsid is not required for formation of infectious particles in this system. Furthermore, the titer of syncytium-inducing units with S*rep*M*env*-tr was $\sim 10^6$/ml in some experiments, more than 10 times higher than that achieved with the two-vector system described above.

TABLE 2. Physical characteristics of infectious particles[a]

Treatment	Syncytia	Single βgal-positive cells
Expt I		
A. None	510	17,000
B. RNase (10 μg/ml, 30°C, RT[b])	510	17,000
C. Pelleted (17,000 × *g*, 75°C)	283	9,350
D. Supernatant of pellet	0	11
Expt II		
A. None	289	8,500
B. Pelleted	138	3,400
C. Triton X-100 (0.1%) - pelleted	0	0
D. Mixture of resuspended pellets from lines B and C above	72	7,950

[a] NIH 3T3 cells were infected with 0.5 ml of 0.45-μm-filtered homogenates of BHK cells that had been electroporated with S*rep*β*gal* plus S*cap*M*env*-tr. The homogenates were centrifuged and/or treated with RNase or detergent as indicated. The NIH 3T3 cells were scored for syncytia and βgal-positive cells 24 h after infection. Since 0.1% Triton X-100 is toxic for cells, Triton-treated homogenates were first pelleted and the pellets were resuspended in Dulbecco modified Eagle medium–5% fetal calf serum. To show that the resuspended pellet of Triton-treated homogenates was free of toxicity, we performed the mixing experiment shown in experiment II, line D.
[b] RT, room temperature.

Vector RNA is intimately associated with membranes of the cells in which it is produced. To see if infectious particles were produced by nonspecific trapping of vector RNA by membrane vesicles formed during dounce homogenization (perhaps analogous to the formation of liposomes), we performed a mixing experiment. BHK cells electroporated with S*rep*β*gal* were mixed with BHK cells electroporated with S*rep*M*env*-tr, and the mixture of cells was then subjected to dounce homogenization. If infectious particles were formed by nonspecific trapping of RNA in cell membranes, one would expect S*rep*β*gal* vector RNA to be trapped in membranes containing the fusogenic MLV Env. For negative and positive controls we used BHK cells electroporated with each vector separately or coelectroporated with both vectors. Cells electroporated with either vector alone did not transmit βgal. Cells coelectroporated with the two vectors gave a βgal titer of 2×10^4/ml, whereas in the separately electroporated but cohomogenized cells the βgal titer was 40-fold lower (5×10^2/ml). This implies that vector RNA is fairly tightly associated with the membranes of the cells in which it is produced and does not indiscriminately complex with membranes during dounce treatment. As an additional control, we measured the syncytium-inducing titers of the coelectroporated and cohomogenized cells: both had syncytium-inducing titers of $\sim 5 \times 10^4$/ml, indicating that infectious particle formation was equivalent in the two preparations. The syncytia almost certainly resulted from transmission and expression of the S*rep*M*env*-tr RNA rather than from fusion from without by Env protein in membranes in the inoculum, because syncytia took ~8 h to appear, the same time it took for the S*rep*β*gal* vector to induce βgal activity.

The infectious particles we observed with SFV and MLV Env-tr vectors closely resemble the phenomenon reported by

Rolls et al. (15) with SFV vectors encoding VSV G protein. In both cases the infectious particles were Triton sensitive, insensitive to RNase, and had a density of 1.09 to 1.13 g/ml. Rolls et al. (15) found that infectivity could be increased several orders of magnitude by sonicating electroporated cells, whereas we found a similar increase following dounce homogenization.

The increase in titer with sonication or homogenization can be interpreted in two ways. One is that the majority of infectious particles are created during sonication or homogenization by a process of random capture of vector RNA in vesicles containing viral Env. A random and artificial process would be consistent with efficient packaging of vector RNAs whether or not they contained a packaging sequence, as was the case in our experiments since the S*cap*M*env* vectors do not contain the region thought to bear the SFV packaging sequence (20). Alternatively, the particles could exist in an immature form inside cells and be released or completed during sonication/homogenization. The latter possibility is favored by our mixing experiment. While the infectious material produced by SFV vectors encoding M*env*-tr was particulate in the sense that it pelleted at 17,000 $\times$ *g* (Table 1), it could consist of very heterogeneous membranous material rather than uniform, virus-like particles.

The fact that our particles were infectious only when we used MLV Env with a truncated cytoplasmic tail is not surprising since removal of the cytoplasmic tail of MLV TM is required for retroviral infectivity (14) and this cleavage is normally performed by the retroviral protease (17) which was not present in our system. It is intriguing that removal of part of the cytoplasmic tail of human immunodeficiency virus type 1 also enhances fusogenicity (6), even though the cytoplasmic tail of lentivirus TM is not cleaved during virus maturation. Our failure to generate infectious particles with vectors encoding chimeric MLV envelopes with SFV E2-derived cytoplasmic tails could have many explanations, including instability of chimeric Env proteins, failure to form particles with these Envs on the surface, or nonfunctionality of chimeric Env molecules.

One possible application for the kind of vector described here is in vaccination against retroviral Env. It has been shown that inoculation of animals with naked, SFV vector RNA can lead to an immune response to vector-encoded proteins (3, 25). This process is presumably inefficient due to rapid degradation of naked vector RNA. More efficient delivery of vector may be achieved by packaging vector RNA in replication-defective SFV particles using the two-vector SFV system, as reported in mice and monkeys (12, 25, 26). Because these particles do not undergo subsequent rounds of replication, however, they may be suboptimal immunogens. Replication-competent alphavirus vaccines have been made by inserting a target gene downstream of a duplicated subgenomic promoter (13). However, these viruses lose the inserted genes fairly rapidly as a consequence of selection against packaging longer RNAs and the tendency of alphaviruses to generate RNAs with internal deletions. A similar approach was recently reported with SFV vectors encoding VSV G protein (16). Replacement of the SFV envelope with that of a retrovirus represents a different approach to engineering an attenuated alphavirus vaccine for retroviral Env. A potential advantage of this approach is that only vectors that retain *env* sequences should be able to spread. While inefficient release of infectious particles would seem to be an important limiting factor in our system, Li and Garoff recently reported that SFV vectors encoding the Moloney MLV capsid can form retrovirus particles that are spontaneously released at titers of $\sim 10^6$/ml (9).

Perhaps the most intriguing aspect of the infectious vectors described here is their primitiveness. Without a capsid, they represent a simplified form of self-replicating RNA capable of limited spread in tissue culture. Spontaneous mutations or intentional modifications that increase infectivity could be selected for on serial passage, and these might shed light on virus evolution as well as enhance the utility of these vectors as vaccines.

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